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14. ABSTRACT

For low-penetrance breast cancer risk alleles it is currently unknown how they lead to predisposition. Here, we study the Mcs5a locus that is associated with breast cancer risk in rats and humans. In our rat model we show that the presence of the resistant genotype of two components of the locus (Mcs5a1, Mcs5a2) down regulates the expression of the Fbxo10 gene in the T cells and that this reduced expression is associated with reduced mammary tumor multiplicity. We show that genetic elements in Mcs5a1 and Mcs5a2 are physically close to each other in the nuclear space. The spatial organization of the locus in primary T cells is conserved between rat and human. We present a model that begins to explain how the Fbxo10 gene could be regulated in T cells.

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INTRODUCTION

Breast cancer susceptibility is a complex, polygenic trait, in which the cumulative effects of low penetrance, high population frequency, risk-altering genetic variants (modifiers) determine the heritable fraction. To be able to construct genetic risk profiles and population-based intervention programs directed to those at highest risk, it is important to identify as many risk alleles as possible. Using whole-genome linkage studies in inbred rat models that vary in susceptibility to carcinogen (DMBA; 7,12-dimethylbenz(a)anthracene)-induced mammary cancer, we found mammary carcinogenesis susceptibility QTL *Mcs5* (Samuelson et al. 2003). Using congenic recombinant inbred lines that have small pieces of the resistant genome introgressed in the susceptible background, *Mcs5* was found to contain at least three distinct loci (*Mcs5a-c*) (Samuelson et al. 2005). *Mcs5a* has been mapped to ultra-fine resolution and was found to be a compound QTL, consisting of two loci (*Mcs5a1*, ~ 30 Kb; *Mcs5a2*, ~80 Kb) that synthetically interact only in *cis* (on the same chromosome) to confer resistance (Samuelson et al. 2007). Human *MCS5A* has essentially the same genetic features as rat *Mcs5a*. Interestingly, in two population-based case-control studies (~12,000 women), the minor alleles of a SNP (single nucleotide polymorphism) in human *MCS5A1* and a SNP in human *MCS5A2* associate significantly with an altered breast cancer risk (Samuelson et al. 2007). These SNPs could either be causative themselves, or be a marker for the causative SNP. This human association study clearly demonstrates the utility of rat models to identify unbiased potential human breast cancer candidates.

Since *Mcs5a* is entirely non-coding, the causative genetic elements will likely involve transcriptional regulation. All genes within 0.5 Mb flanking the QTL are expressed at similar levels in the mammary glands in susceptible and resistant congenic animals (Samuelson et al. 2007). However, *Fbxo10* and *Frmpd1*, the genes transcriptionally starting off in *Mcs5a*, are differentially expressed in thymus and spleen, respectively. However, only the expression level of *Fbxo10* in the thymus is correlated with mammary carcinogenesis susceptibility (unpublished). *Mcs5a1* and *Mcs5a2* also need to be both present to reduce the expression in the thymus. Flow cytometry experiments revealed that the *Fbxo10* differential expression is limited to T cells (unpublished). In addition, a mammary gland transplantation assay indicated that there is a host effect on mammary carcinogenesis, suggesting a mammary cell-non autonomous effect of the *Mcs5a* locus on mammary carcinogenesis susceptibility (Samuelson et al. 2007).

We hypothesize that in T cells, genetic elements in *Mcs5a1* and *Mcs5a2* are looping over to physically interact in order to regulate the expression of the *Fbxo10* gene, which thymic expression level is correlated with mammary carcinogenesis susceptibility.

BODY

Training

Lab Meetings and Seminars at the University of Wisconsin (SoW Task 1)

As part of my postdoctoral training, I participated by attending and presenting in the Gould lab meetings and in the student/postdoc seminar series organized by the McArdle Lab for Cancer Research. On a weekly basis, I attended seminars given by invited specialists on diverse cancer biology related topics, including transcriptional regulation, biostatistics, genetics, genomics, and more.

Visit Dr. Job Dekker's lab (SoW Task 2)

The chromatin conformation capture (3C) technology is a crucial procedure for understanding how the *Mcs5a* locus is organized in the nucleus to regulate the expression of *Fbxo10*. The 3C assay was invented by Dr. Job Dekker in 2002 (Dekker et al. 2002). I visited Dr. Dekker's lab at the University of Massachusetts Medical School (Worcester, MA) to obtain hands-on training in the 3C assay. Following the visit, I have successfully implemented the 3C assay in the Gould lab. Using 3C, I profiled the *Mcs5a* region in various rat cell types and human cell lines.

Scientific Meetings (SoW Task 3)

I took part in an international scientific meeting, Keystone Symposia 'Complex Traits: Biological and Therapeutic Insights', Santa Fe, NM, held February 29 – March 5, 2008. I was assigned an oral presentation and a poster presentation. My abstract was awarded a Keystone Symposia Travel Scholarship.

I participated with a poster presentation in the Era of Hope DoD BCRPM meeting, Baltimore, MD, held June 25 – 28, 2008.

Mentoring Committee (SoW Task 4)

Although a formal meeting with the entire mentoring committee has not taken place yet, I had regular discussions with the members separately. I'm having discussions with my primary mentor, Dr. Michael Gould, at least once a week. I presented my work at the Transcriptional Mechanisms seminar series organized by Dr. Emery Bresnick, which was followed by a discussion. I'm having regular (~monthly) discussions with Dr. Sündüz Keles. Discussions with Dr. William Dove take place when needed.

Research

The 3C assay (SoW Task 1)

To identify a physical interaction between genetic elements in *Mcs5a1* and *Mcs5a2*, implementation of the chromatin conformation capture (3C) assay is essential. In collaboration with the lab of Dr. Job Dekker, who invented the 3C assay in 2002 and has a broad experience in using it (Dekker 2006), the 3C assay was established in the Gould lab (SoW Task 1a). To capture chromosomal interactions, cells are fixed using formaldehyde. The extracted fixed chromatin is digested with a restriction enzyme and religated in a strongly dilute fashion. In this procedure the ligation of genetic elements that were glued together by formaldehyde fixation is favored over ligation of random elements. Following reversal of the crosslinks, the ligation frequency of two elements of

interest is determined quantitatively. The measurements will be relative to a fully digested and randomly ligated control template containing all restriction fragments of interest in equal molarity. To investigate the *Mcs5a1-Mcs5a2* interaction a fixed fragment in *Mcs5a1* was chosen and the relative interaction frequency to all restriction fragments in *Mcs5a2* was determined. This results in a regional profile in which fragments close to the fixed fragments give a high relative interaction frequency, due to random ligation events. Such random events decrease with increasing genomic distance. Local peaks in the profile are indicative of a physical interaction.

The 3C assay was applied to our rat models to address three fundamental questions about the structural organization of the *Mcs5a* locus: 1. Does the structural organization support a physical interaction between an element in *Mcs5a1* and an element in *Mcs5a2*? 2. Does the susceptible or resistant genotype have an effect on the structural organization of *Mcs5a*? 3. Is the structural organization of *Mcs5a* different between various tissues / cell types?

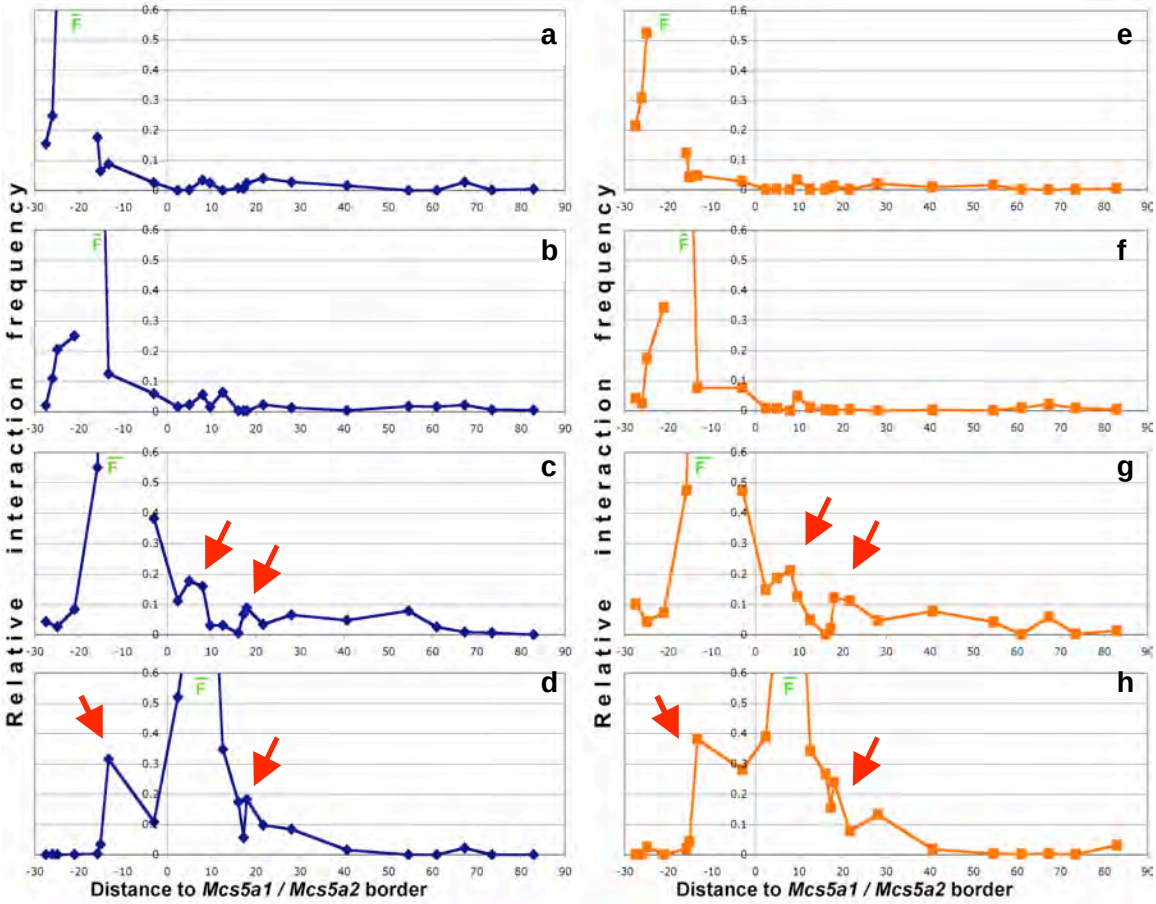

Figure 1: 3C profiles of the rat *Mcs5a* locus in splenic T cells of susceptible line WF.WKy (a-d) and resistant congenic animals WW (e-h). The fixed fragments used are indicated by a green bar. Each point is the average of at least three measurements on 3C template pools of six rats per genotype. Red arrows indicate areas of potential looping.

To answer the first question, if *Mcs5a1* is actually in close proximity to *Mcs5a2*, the 3C assay was used on (splenic) T cells of the susceptible WF.WKy strain. This cell type has been shown to differential express the *Fbxo10* gene and is considered the cell type of action, as described below (SoW Task 2b). The 3C assay was performed with three

different fixed fragments in *Mcs5a1* that were probed for interactions with all working restrictions fragments in *Mcs5a2*. The fixed fragments in *Mcs5a1* were chosen to be close to the *Fbxo10* promoter and putative regulatory elements. Figures 1a and 1b show the chromatin profiles for the first two fixed fragments, closest to the *Fbxo10* putative proximal promoter, as determined by start site analysis, described later (not in SoW). These two fixed fragments do not show outstanding interactions with any elements in *Mcs5a2*. In figure 1c the fixed fragment is slightly shifted towards putative upstream regulatory elements of the *Fbxo10* gene. Probing all *Mcs5a2* restriction fragments with this fixed fragment yielded locally enhanced interaction frequencies with at least two elements in *Mcs5a2*, close to the *Mcs5a1/Mcs5a2* border. This 3-way interaction was confirmed by using one of the two *Mcs5a2* interacting elements as the fixed fragment and scanning both ways, as shown in figure 1d.

To investigate the second question about the effect of the susceptible or resistant genotype on putative looping, rats of the susceptible line (WF.WKy), susceptible congenic lines (*Mcs5a1* and *Mcs5a2*), and the resistant congenic line (WW) were used (SoW Task 1b). Figures 1e-h show the chromatin profiles of the *Mcs5a* locus of splenic T cells of the resistant congenic line WW, as determined by 3C using the same fixed fragments as in figures 1a-d. Besides some minor differences, the chromatin profiles of T cells of the susceptible line WF.WKy and the resistant line WW did not differ significantly. Accordingly, the profiles for the susceptible congenic lines *Mcs5a1* and *Mcs5a2* were not found to differ either (not shown), leading to the conclusion that the structural organization of the *Mcs5a* locus is not affected by the susceptible or resistant genotype.

By determining the structural organization of *Mcs5a* in different cell types in our rat models, the third fundamental question was answered. Figures 2a-c display the 3C profiles for splenic non-T cells, splenic T cells, and mammary gland, respectively. The splenic non-T cell population primarily contains B cells, and monocytes. Both these cell types, and the mammary gland have been shown not to differentially express the *Fbxo10* gene, which is in contrast to T cells, as described later (SoW Task 2b). Regardless of the *Fbxo10* expression differences, the genetic elements in the 3-way interaction are identical in these three cell types, although the signal intensity is enhanced in the mammary gland. This result led to the conclusion that the chromatin structure of *Mcs5a* may not be a direct determinant of the differential expression of the *Fbxo10* gene seen in T cells, but the structure may facilitate transcriptional regulation by certain genetic elements yet to be determined.

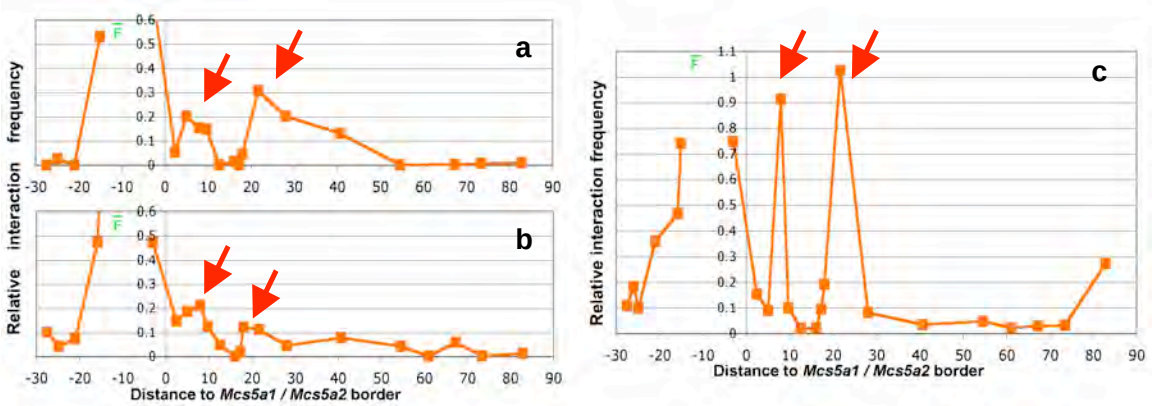

Figure 2: 3C profiles of the rat *Mcs5a* of resistant congenic animals WW in splenic non-T cells (a), splenic T cells (b), and mammary gland (c). The fixed fragments used are indicated by a green bar. Again each point is the average of at least three measurements on 3C template pools of 4-6 rats per genotype. Red arrows indicate areas of potential looping.

Finally, the 3C technology was applied to various human cell lines (SoW Task 1c). Human *MCS5A* has the same genetic features as rat *Mcs5a*. In both human orthologous regions of *Mcs5a1* and *Mcs5a2*, novel breast cancer risk alleles were identified in a large case-control study (Samuelson et al. 2007). The question now becomes: Besides the sequence, the genetic features, and the association with breast cancer risk, is the structural organization also conserved between rat and human *Mcs5a*? Figures 3a-c display the chromatin structure of *MCS5A* of a cervical carcinoma cell line (HeLa), a mammary carcinoma cell line (MCF-7), and a leukemic T lymphocyte cell line (JURKAT). These profiles were determined using a fixed fragment that includes the promoter of the *FBXO10* gene. In the rat, no clear interactions were detected using fixed fragments with the *Fbxo10* promoter, as described above (Fig. 1a,b,e,f).

It turns out that in all three cell lines many interactions were picked up, indicative of more condensed chromatin, possibly due to the cancerous nature of the cell lines (Holloway and Oakford 2007). When primary T cells were used, the human *MCS5A* chromatin profile did not show any obvious interactions (Fig. 3d), which resembles the profile in rat T cells (Fig. 1a,b,e,f). Similarly, when the fixed fragment was shifted towards putative upstream regulatory elements, two areas of enhanced local interaction frequency close to the *MCS5A1/MCS5A2* border could be picked up again, just like in rat T cells. Primary T cells nicely reflected the structural organization of *MCS5A*, however, human cell lines of cancerous origin do not fully reflect the structural organization of the *MCS5A* locus. Therefore, these cell lines might not be suitable to model the gene regulatory properties of the *MCS5A* locus.

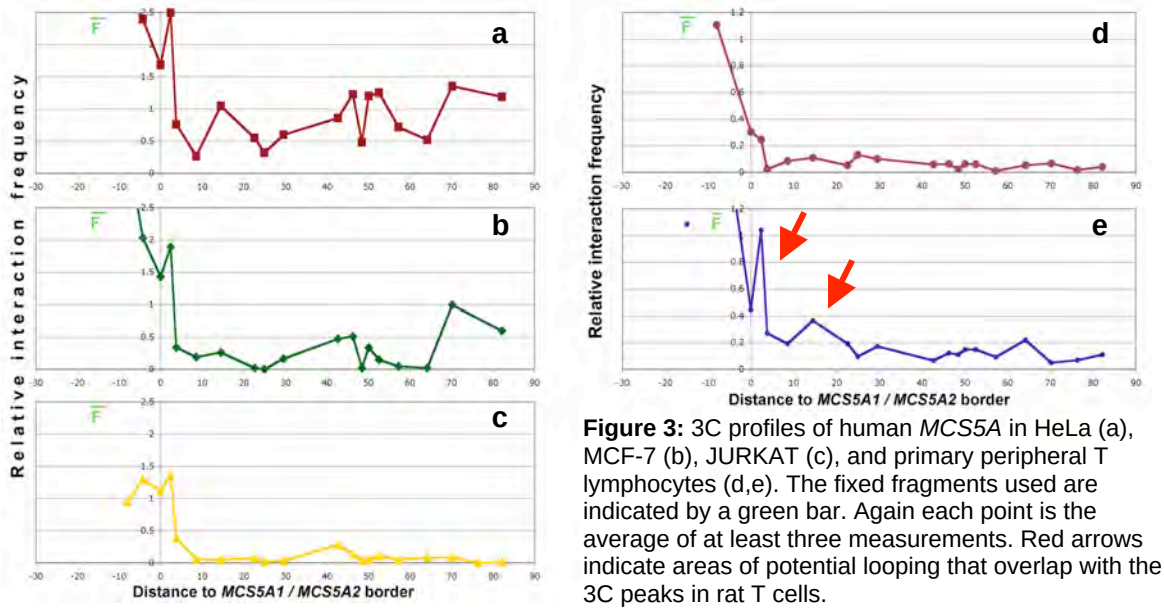

Figure 3: 3C profiles of human *MCS5A* in HeLa (a), MCF-7 (b), JURKAT (c), and primary peripheral T lymphocytes (d,e). The fixed fragments used are indicated by a green bar. Again each point is the average of at least three measurements. Red arrows indicate areas of potential looping that overlap with the 3C peaks in rat T cells.

Regulation of gene expression (SoW Task 2)

As much of the structural organization of the *Mcs5a* locus is currently known, the next step would be to understand how the locus would regulate gene expression that ultimately predisposes to breast cancer. Previous expression analysis of all genes within

1 Mb of the *Mcs5a* locus in the rat mammary gland of susceptible (WF.WKy) and resistant congenic (WW) animals yielded no expression differences. A co-worker in the Gould lab, Dr. David Samuelson, proceeded with expression analysis in other tissues of WF.WKy and WW animals and found the *Fbxo10* gene to be differentially expressed in the thymus and the *Frmpd1* gene in the spleen (SoW Task 2b). When these two genes were profiled in the thymus and spleen of the two susceptible congenic lines (*Mcs5a1, Mcs5a2*) that just have *Mcs5a1* or *Mcs5a*2 of the resistant genotype, only the expression level of *Fbxo10* in the thymus appeared to be correlated with the mammary carcinogenesis susceptibility phenotype. In other words, the *Mcs5a1-Mcs5a2* interaction is required for both down regulation of thymic *Fbxo10* expression and reduced tumor multiplicity in our carcinogenesis model (not shown).

The thymus consists mainly of T lymphocytes that could be expressing the CD4 receptor (CD4+), the CD8 receptor (CD8+), both receptors (double positive), or none of the receptors (double negative). Using flow cytometry these cell types were separated from the thymus of WF.WKy and resistant WW animals and their *Fbxo10* expression level was determined. Single positive CD4+, single positive CD8+, and double positive thymocytes differentially expressed the *Fbxo10* gene, whereas double negative thymocytes did not. When isolated from the spleen, single positive CD4+ and CD8+ T lymphocytes persisted in their differential *Fbxo10* expression, whereas other cell types isolated from the spleen, such as B lymphocytes, and monocytes, did not (not shown). At this point the T lymphocytes are considered the cell type of action (SoW Task 2b).

It should be noted that the expression level of both human *FBXO10* and *FRMPD1* have been determined in the above mentioned human cell lines that were also used for 3C (SoW Task 2a). However, since the *MCS5A* chromatin profiles of these cancerous cell lines are not representative of the situation in primary cells, the original question if the chromatin structure influences gene expression could not be answered. Moreover, in the rat, the chromatin structure of the *Mcs5a* locus did not differ from cell type to cell type, whereas the differential expression of *Fbxo10* was found to be cell type dependent. Thus, the chromatin structure is not directly influencing *Fbxo10* expression, but might facilitate gene regulation by the specific regulatory elements.

In order to reveal where these regulatory elements that control the expression of *Fbxo10* in T lymphocytes could be located, it is essential to find its transcriptional start site (not in SoW). An assay to identify transcriptional start sites of transcripts is the 5'RACE assay (RLM-RACE: RNA Ligase Mediated-Rapid Amplification of cDNA Ends; Ambion). Briefly, total RNA was isolated from rat spleen and thymus tissue. After removal of the 5'CAP structure an RNA-adapter was ligated to the 5'ends of mRNAs. Following an RT (reverse transcriptase) reaction to make cDNA, a PCR reaction with a primer annealing to the translational start codon (ATG)-containing exon and a universal primer annealing to the 5'adapter was performed. This procedure potentially leads to the amplification of all transcriptional start sites of the *Fbxo10* transcripts. These start sites in rat thymus and spleen tissues were found to be located close to the only CpG island of the *Mcs5a1* locus (Fig. 4a). The same procedure on human thymus and spleen total RNA (Ambion) revealed the same transcriptional start site location for the human *FBXO10* gene. The start site of the *FBXO10* gene in immune tissue has never been found in the CpG island located close to the *MCS5A1/MCS5A2* border (Fig. 4a), whereas in human breast total RNA this transcriptional start site location was identified for the *FBXO10* gene. These findings implicate that the active proximal promoter for the *FBXO10* gene in T lymphocytes is most likely located close to CpG island in *MCS5A1*.

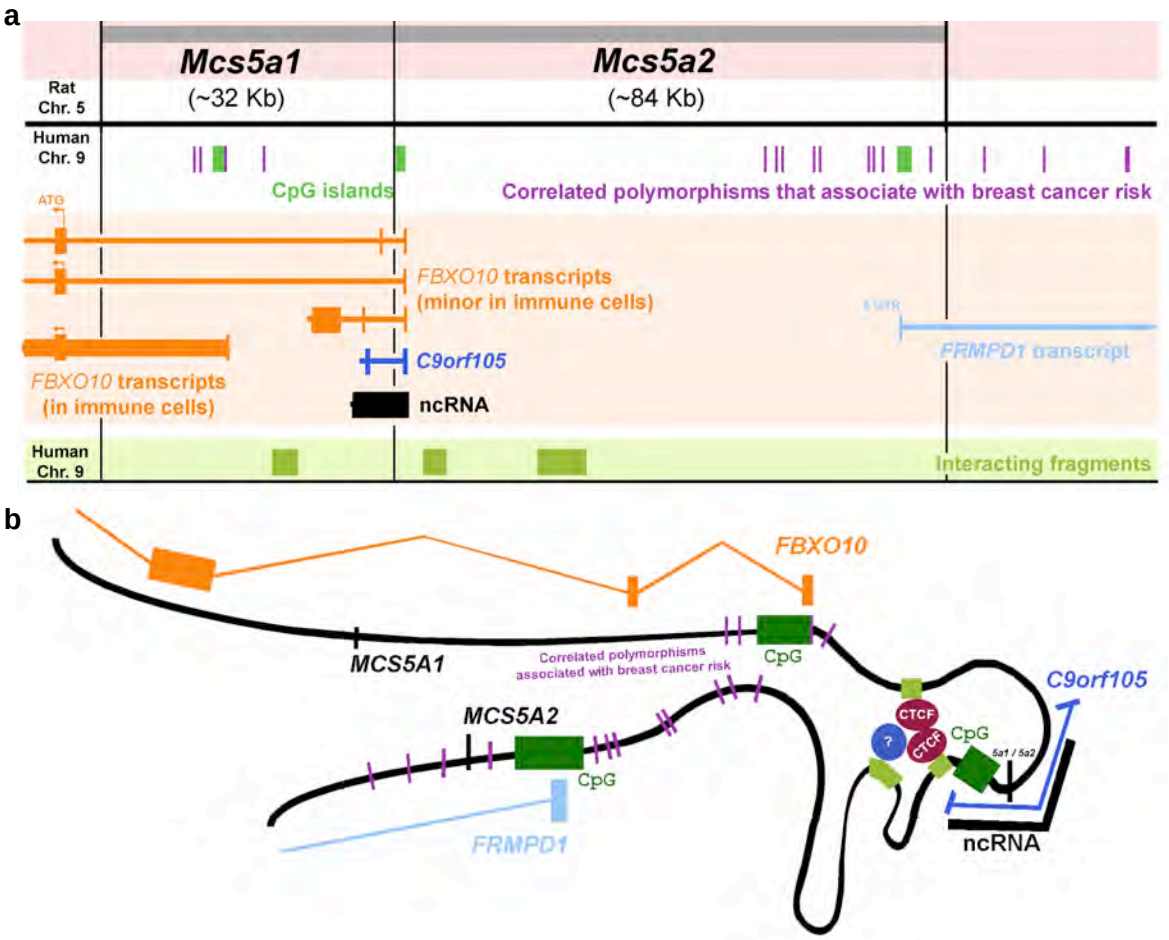

Figure 4: The *MCS5A* locus in a flat representation (a) and in a folded representation as determined by the 3C experiments (b). The *FBXO10* transcripts, as annotated in the UCSC browser are shown in orange. Note that there are two major start site areas associated with CpG islands (in dark green). The predominant *FBXO10* transcript in immune cells is displayed in bold. The *FRMPD1* transcript is shown in light blue. Its start site is associated with a third CpG island (in dark green) at the end of the *MCS5A2* locus. The correlated polymorphisms that associate with risk are shown as purple bars. The interacting elements in the 3C assay are shown in light green. Other small transcripts that start off from the promoter close to the *MCS5A1/ MCS5A2* border are shown in blue (*C9orf105*) and black (ncRNA).

The model

Integration of the structural organization data, the gene expression data, and the start site analysis resulted in a model that might explain how the *Mcs5a* locus could regulate the expression of *Fbxo10* in T lymphocytes (SoW Task 2c). Coincidently (or not), the human correlated polymorphisms in *MCS5A1* that associated with breast cancer risk in women, are located within and directly surrounding the CpG island in *MCS5A1* from which *FBXO10* is transcriptionally initiated in T lymphocytes (Fig. 4a). These polymorphisms are now thought to directly affect *FBXO10* transcriptional initiation in human T lymphocytes, which might partially explain risk mediated by *MCS5A1*.

Figure 4b presents a model for the structural organization of the *MCS5A* locus. If the interacting fragments (indicated in light green in figure 4a) are drawn together by a protein complex (Fig. 4b), the correlated polymorphisms in *MCS5A1* and *MCS5A2* are physically closer in the nuclear space compared to their seemingly larger genomic distance. This organization of the locus might eliminate the action of the promoter

associated with the CpG island close to the *MCS5A1/MCS5A2* border and allow for the transcriptional initiation and regulation of the *FBXO10* gene from the promoter associated with the CpG island in *MCS5A1*.

Future work

The main focus of the near future experiments will be to understand what the function of the elements that bear the genetic variants is. The plan is to proceed with Task 3 of the SoW, which involves screening of all alleles of the risk-associated polymorphisms for transcriptional and enhancer activity using *luciferase* reporter assays. This data is anticipated to nicely fit to the model discussed above. Ultimately, the actual transcription factors will be identified that regulate the expression level of the *FBXO10* gene in T lymphocytes, which expression level is associated with mammary carcinogenesis susceptibility in our rat model.

KEY RESEARCH ACCOMPLISHMENTS

Training:

- Actively participated in the Gould lab meeting/journal club (SoW Task 1)
- Actively participated in McArdle Lab student/postdoc seminar series (SoW Task 1)
- Attended seminar series on a variety of cancer biology and related topics (SoW Task 1)
- Visited Dr. Job Dekker's lab (UMass Medical School, Worcester, MA) to learn the 3C technology (SoW Task 2)
- Presented at an international scientific meeting: Keystone Symposia 'Complex Traits: Biological and Therapeutic Insights', Santa Fe, NM (SoW Task 3)
- Presented a poster at the Era of Hope DoD BCRPM meeting, Baltimore, MD (SoW Task 3)
- Regular discussions with members of the mentoring committee (SoW Task 4)

Research:

- Established the 3C assay in the Gould lab, after visiting the Dekker lab at UMass Medical School, Worcester, MA (SoW Task 1a)
- Applied 3C technology to various tissues of rats of the susceptible line (WF.WKy), the susceptible congenic lines (*Mcs5a1* and *Mcs5a2*), and the resistant congenic line (WW) (SoW Task 1b)
- Applied 3C technology to various human cell lines (SoW Task 1c)
- Determined expression levels of *FBXO10* in human cell lines (SoW Task 2a)
- Determined expression levels of *Fbxo10*, *Frmpd1* in various tissues of rats of the susceptible strain (WF), susceptible congenic lines (*Mcs5a1* and *Mcs5a2*), and the resistant congenic line (WW) (SoW Task 2b)
- Designated T lymphocytes as the cell type of action
- Determined transcriptional start site of *Fbxo10* in rat and human (not in SoW)
- Concluded 3C experiments, generated a model as a hypothesis for regulation of the *Fbxo10* gene by the *Mcs5a* locus in rats and humans (SoW Task 2c)

REPORTABLE OUTCOMES

- Abstract Keystone Symposia 'Complex Traits: Biological and Therapeutic Insights', Oral and Poster presentation

- Traveling Scholarship Award Keystone Symposia 'Complex Traits: Biological and Therapeutic Insights'

- Abstract Era of Hope DoD BCRPM Meeting, Poster presentation

CONCLUSION

The mammary cancer susceptibility locus *Mcs5a* was found to possess a folded configuration in the nucleus. This folded configuration is not influenced by the susceptible and resistant genotype, and is not strikingly different between cell types. However, the structural organization of the locus is conserved between rat and human primary T cells. It is thought to physically bring closer the regulatory genetic elements that are marked by the correlated polymorphisms that associate with breast cancer risk from our case-control study.
The expression level of the *Fbxo10* gene in the thymus was found to associate with mammary carcinogenesis susceptibility in our model system. The *Fbxo10* expression difference between the susceptible and resistant animals was found to take place in the T cells. The main focus in order to explain how genetic variants in both rats and humans predispose to breast cancer the main focus now becomes to understand how the regulatory genetic elements regulate the expression of the *Fbxo10* gene in T lymphocytes in the context of their spatial organization.

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APPENDICES

none